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Evaluation of Cathepsin D Immunostaining in Colorectal Adenocarcinoma

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Background and Objectives: Cathepsin D (CD), an estrogen-regulated lysosomal protease, has been detected in a variety of tissues. CD expression has been correlated with the invasive potential of breast cancer, acting as an autocrine mitogen or as a protease that degrades the extracellular matrix. The role of CD expression in predicting prognosis or invasive potential in colorectal carcinomas is mostly unknown.
Methods: CD immunohistochemical expression was studied in 60 surgical specimens of colon adenocarcinomas. A three-step avidin biotinylated, horseradish immuno-peroxidase (ABC-HRP) staining technique was performed on 4 μm paraffin-embedded tissue sections with a polyclonal antibody to CD.
Results: Carcinoma cells showed positive CD immunostaining in 41.6% of adenocarcinomas (50%, 43.7%, 37.5%, and 25% of Dukes' Stage A, B, C, and D, respectively). Nonneoplastic stromal cells demonstrated positive staining in 68.3% of the adenocarcinoma specimens (37.5%, 62.5%, 91.6%, and 75% of Stage A, B, C, and D, respectively). Patients with colorectal carcinomas exhibiting simultaneously negative and positive CD expression in malignant and stromal cells, respectively, had a worse 5-year overall survival ($P < 0.05$). The mean 5-year survival of the 16 patients overexpressing CD in nonneoplastic stromal cells (>15% of stromal cells positive for CD) was significantly worse in comparison with the rest of the adenocarcinomas (n = 44) (27.6 ± 4.6 vs. 46 ± 2.7 months, respectively, $P < 0.01$).
Conclusions: Expression of CD immunoreactivity by the stromal cells may be associated with a more invasive phenotype. Therefore, CD expression in tumor and stromal cells may serve as an important indicator of progression and guide postoperative treatment.
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KEY WORDS: **colorectal cancer; adenocarcinoma; cathepsin D; protease; immunohistochemistry; Dukes' stage; prognosis; treatment**

INTRODUCTION

Colorectal cancer is second in incidence, after carcinoma of the lung, in many Western countries. An improved understanding of the fundamental genetics and biology in colorectal cancer may be the basis in achieving new approaches to its prevention and treatment. In addition, prognosis of patients seems to be related mainly to the control of both progression and metastasis of the malignant tumor. In the metastatic process, proteolytic enzymes play an important role in mediating passage of the malignant cell through the cell membrane [1].

The cathepsins are umbiquitous lysosomal proteases and are classified both functionally and according to their

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active site [2–4]. Increasing experimental evidence indicates that cathepsin D (CD), a lysosomal acid protease (aspartylendopeptidase) mainly involved in intracellular protein catabolism [5], also may be implicated in tumor invasion and metastasis [6].

In 1980, the role of CD in breast cancer was first reported by Westley and Rochefort [7], who found that a glycoprotein secreted by a hormone-sensitive breast cancer cell line was induced by estrogen. They described it as a 52-kilodalton (Kd), estrogen-inducible protein in the MCF-7 breast cancer line [8]. This protein also was shown to be mitogenic and to be inhibited in certain breast cancer cell lines by the antiestrogen tamoxifen [9,10]. Subsequent studies revealed that the 52-Kd protein was the precursor form of the protease CD [11]. CD exists in three forms: the secreted form is the 52-Kd protein, which is enzymatically inactive and is converted to the intermediate, active, 48-Kd enzyme, and the mature, active 34-Kd and 14-Kd enzyme dimer forms [12–14].

CD has been detected in a variety of tissues [15,16] and its presence in breast cancer has been extensively correlated to the poor prognosis of the patients [10,11,17–24]. Except for tumor cells, stromal cells contain CD in their cytosol, and several investigators have suggested that the cathepsins derived from inflammatory and stromal cells play a more important role in cancer progression than those from carcinoma cells [25–27].

The role of CD expression in predicting prognosis or invasive potential in colorectal carcinomas is mostly unknown. This study examines the prevalence of CD immunohistochemical expression in the primary colorectal adenocarcinoma and in the lymph node metastasis, evaluating the expression of this putative tumor marker in cancer and reactive stromal cells as well as its possible prognostic significance.

MATERIALS AND METHODS

The CD immunohistochemical expression was studied on 60 surgical specimens of colon adenocarcinoma. All selected patients received conventional surgical management for colorectal cancer at the surgery departments in Hippocration General Hospital (Athens Medical School, Greece), from 1987 to 1988. The mean age of patients was 65.7 ± 12.7 years (range 18–81 years), and the male/female ratio was 26/34 (0.76/1). Most adenocarcinomas of this survey were located in the rectum (n = 29) and sigmoid (n = 10) (48% and 17% of the total, respectively). The carcinomas were classified according to sex, age, location, degree of differentiation, Dukes' stage, and a 60-month follow-up period.

A three-step immunoperoxidase staining technique was used on paraffin-embedded, 4-μm-thick tissue sections from primary colorectal tumors, adjacent uninvolved mucosa, and malignantly infiltrated lymph nodes. Another section was stained with hematoxylin-eosin (HE) for morphological investigation. After deparaffinization through graded alcohols, endogenous peroxidase activity was blocked by incubating the slides in 0.1% hydrogen peroxide in methanol for 20 minutes. Immunostaining was performed using the avidin-biotinylated horseradish peroxidase (ABC-HRP) method (Dakopatts, Denmark). As a primary antibody, a polyclonal antibody to cathepsin D (Dako, Glostrup, Denmark) was used at a dilution of 1/250 with 1 1/2 hours incubation. Aminoethylcarbazole was used as the chromogen with Meyer's hemotoxin counterstain. Tumor sections subjected to the whole procedure, except for incubation with the primary antibody, were used as ''negative'' controls. Breast cancer sections previously positive for CD were used as positive controls.

All immunostained slides were analyzed and scored in a blinded fashion by two different observers without knowledge of histological type, grade, stage, or survival data. In each section, at least 10 high power fields (×400) were examined under light microscopy, individual cells were counted, and the mean percentage of CD positive cancer cells was calculated among all the malignant cells observed within the primary lesion. The positivity of cancer cells was evaluated differently from that of stromal cells present within or immediately adjacent to the tumor. CD expression was considered positive when tumor cells were stained, irrespective of the percentage of positive cells. CD immunohistochemical expression in nonneoplastic stromal cells was also evaluated. It was considered positive if CD was expressed in >5% of the stromal cells. Overexpression of CD at stromal cells was considered when >15% of stromal cells were stained.

For statistical analysis, Student's *t*-test and Chi-square tests were applied. All results were considered at the 5% level of statistical significance.

RESULTS

CD immunohistochemical expression was a typically cytoplasmic and coarsely granular staining in neoplastic cells of the primary tumor and the metastatically invaded lymph nodes as well as in inflammatory cells of the stroma and adjacent uninvolved mucosa (Fig. 1). Macrophages and polymorphonuclear leukocytes that infiltrated in and around the carcinoma tissue were intensely stained for CD (Figs. 2,3).

Overall, CD expression in carcinoma cells was present in 25 of the 60 evaluated cases (41.6%). The percentage of CD positive tumor cells in all cases was >10% of all the malignant cells observed within the primary lesion. Stromal cells demonstrated positive immunostaining (any degree of CD positivity) in 41 cases (68.3%). Overexpression of CD at stromal cells (>15% of stromal cells showed CD positivity) was observed in 16 cases (26.1%), 13 patients with Dukes' C adenocarcinoma and

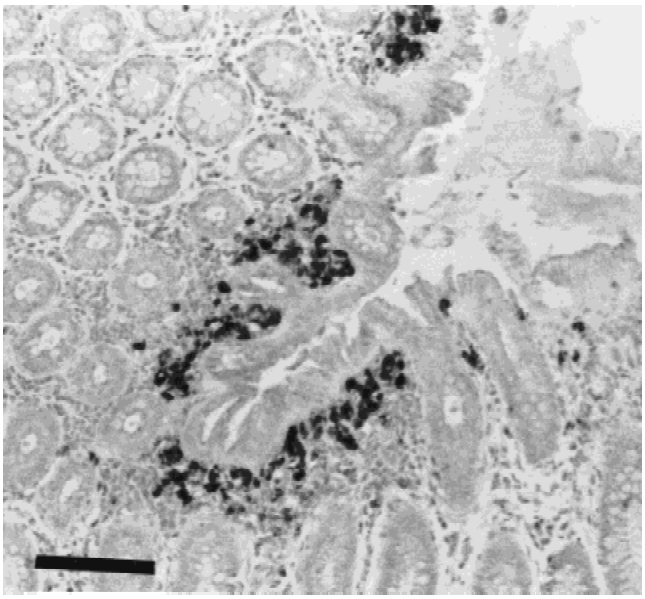

Fig. 1. Immunohistochemical detection of cathepsin D in a colon adenocarcinoma (bar indicates a distance on the histopathologic slide of 0.05 mm).

3 with Dukes' D. Higher CD immunopositivity frequency in cancer cells occurred in stages A and B tumors (50% and 43.7%, respectively). Stages C and D tumors demonstrated the highest incidence of stromal cell CD positivity (91.6% and 75%, respectively), (Table I). None of these differences reached the level of statistical significance. CD immunopositivity in tumor or cancer cells was not correlated with patients' age, sex, or other examined parameters.

The immunostaining intensity and the distribution of CD positive carcinoma cells varied in the same carcinoma tissue. A more homogeneous pattern of CD immunostaining was observed in Dukes' A and B tumors where CD was expressed in tumor cells of both the main tumor mass and the margins of invasion. The most intensely stained and the highest percentage of CD positive tumor cells in Dukes' C and D tissue specimens were frequently aggregated at the advancing margin of the invasion front rather that at the center of the carcinoma tissue. CD positive inflammatory cells on the primary tumor were mainly concentrated at the stromal border of the invasion front of carcinoma tissue irrespective of the stage of the tumor.

Malignantly infiltrated lymph nodes were tested with regard to CD and lymph node metastatic foci from all the CD positive Dukes' C and D (9 of Dukes' C and 1 of Dukes' D) tumors stained positively for CD. The CD expression was proportional and staining intensity to that of the primary tumor location. Nevertheless, lymph node tumor cells from eight CD negative Dukes' C and two CD negative Dukes' D carcinomas showed positive immunostaining. Twenty of the 24 Dukes' C (83.3%) and all Dukes' D cases demonstrated positive immunostaining at stromal cells of the invaded lymph nodes. The percentage and the staining intensity of CD positive stromal cells were always higher in the metastatic lymph nodes than in the primary tumor.

In terms of prognosis, Dukes' stage correlated significantly with overall survival ($P < 0.001$) (data not shown). The absence of CD immunoreactivity in cancer cells showed a clear trend to a worse overall survival ($x^2 = 3.2$, $0.05 < P < 0.10$) (Table II). Similarly, patients with carcinomas with CD positive expression at stromal cells had a worse 5-year survival ($x^2 = 3.39$, $0.05 < P < 0.10$) (Table III). Patients with synchronous cancer cells CD negative and stromal cells CD positive expression had a significant prognostic disadvantage over the rest ($x^2 = 4.21$, $P < 0.05$). (Table IV) The mean survival of the 16 patients (13 Dukes' C and 3 Dukes' D) with overexpression of CD at stromal cells (>15% of stromal cells CD positive) was significantly shorter in comparison with all the others ($n = 44$) (27.6 ± 4.6 vs. 46 ± 2.7 months, respectively, $P < 0.01$).

DISCUSSION

CD is known mostly as an estrogen-regulated enzyme expressed mainly in breast cancer [7]. Elevated amounts of CD at tumor cytosol have been found in ovarian cancer tissue [28] and in thyroid tissue [29]. As far as gastrointestinal malignancies are concerned, cathepsins seem to play an important, although mostly unknown, biological role at the progression of gastric cancer [30–34]. Even if several studies have previously demonstrated the influence of estrogen in colon cancer [35,36], very little is known about the role of CD as a putative tumor marker in this malignancy. Our study confirms the presence of CD in tumor and stromal cells in colorectal adenocarcinoma, in keeping with the few studies in the international literature concerning CD expression in this cancer [37–40].

Galandiuk et al. [37], using immunoassay, showed increased CD levels in neoplastic colon tissues with respect to normal colon regardless of the cells expressing this protease [37]. Valentini et al. [40], performing immunohistochemistry with a monoclonal antibody, detected CD in tumor and stromal cells in colon cancer specimens [40]. These findings along with our results are in agreement with other immunohistochemical studies in which stromal cells can express CD [41,42]. A 15% cutoff point was also used to evaluate the overexpression of CD in stromal cells, as described in recent studies [40,42]. The immunohistochemical approach has the advantage of precise and selective tissue localization of the antigen and easily can be used in order to discriminate the CD expression in stromal cells from that in cancer cells.

The normal epithelial cells adjacent to the tumor mass in the examined cases were negative for CD as in the

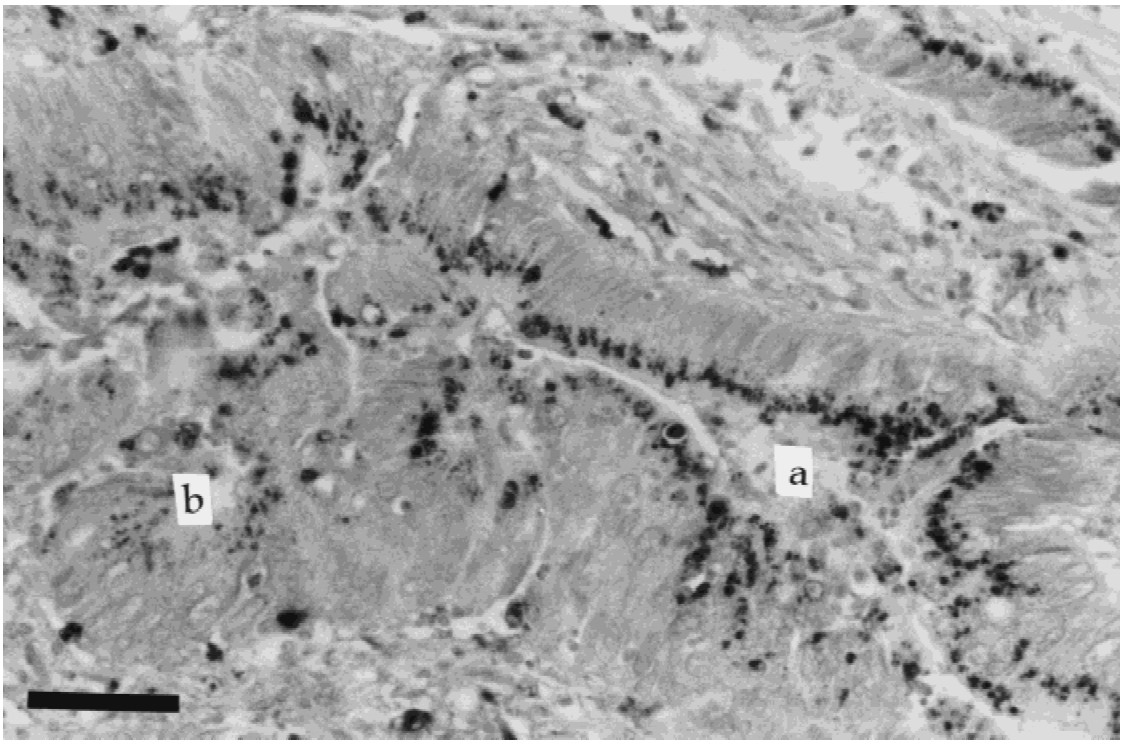

Fig. 2. Cathepsin D immunopositivity in cancer **(a)** and stromal **(b)** cells of a sigmoid adenocarcinoma (see Fig. 1 for bar indication).

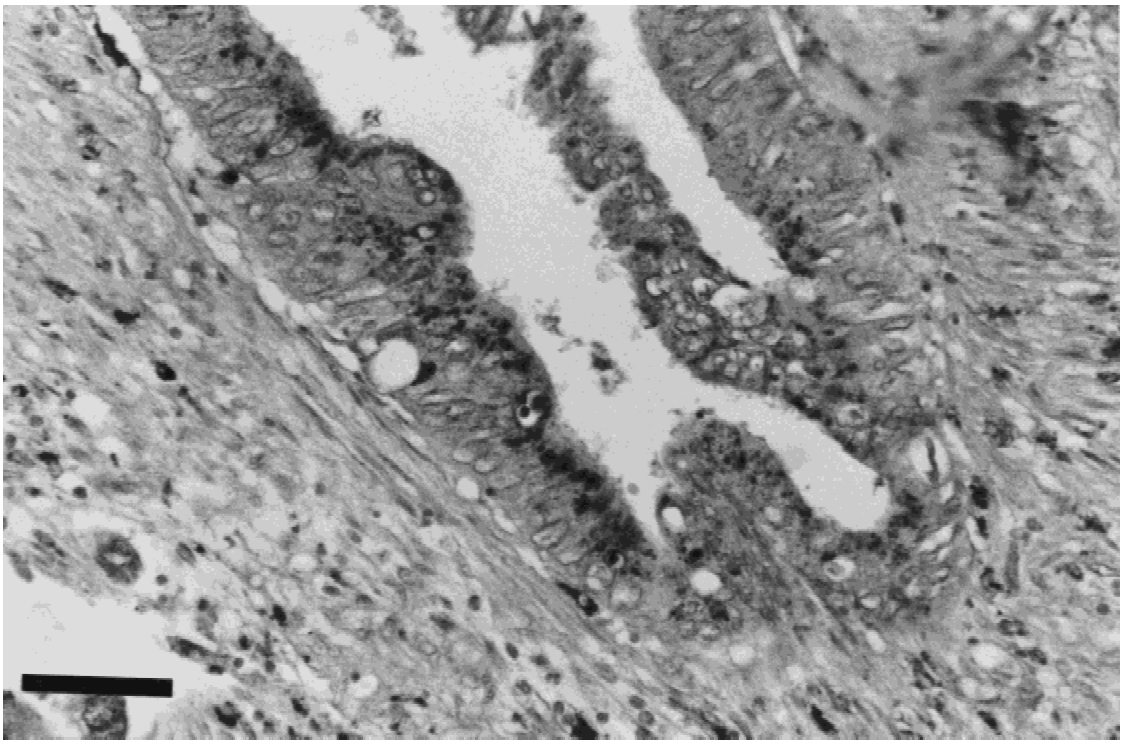

Fig. 3. Cathepsin D immunoreactivity in stromal cells of adjacent colonic mucosa (see Fig. 1 for bar indication).

TABLE I. Incidence of Cathepsin D Immunopositivity in Colorectal Adenocarcinoma: Expression in Carcinoma and Stromal Cells

Dukes' stage n = 60	Cathepsin D expression			
	Carcinoma cells		Stromal cells	
A, n = 16	8/16	50%	6/16	37.5%
B, n = 16	7/16	43.7%	10/16	62.5%
C, n = 24	9/24	37.5%	22/24	91.6%
D, n = 4	1/4	25%	3/4	75%

previous study [40]. The absence of staining in normal mucosa is in agreement with the significantly lower cytosol levels of CD in normal colon with respect to those in neoplastic colon in the previously mentioned cytosol assay [37]. A cutoff point was not used to evaluate CD in colon cancer cells in the present study due to the negative CD staining in the normal colonic mucosa. Meanwhile, no cutoff point has been used in the recent studies regarding CD expression at tumor cells in colon cancer [37,40]. The discrepancies in the incidence of CD immunohistochemical expression in our series and the previous ones may partially reflect differences in number of cases, experimental conditions, and types of antibodies used.

Although CD is reported to be hormonally induced in breast cancer, CD expression in colorectal cancer did not seem to be associated with a patient's sex or age, which is related to endocrine status (i.e., menopause) [37]. The increased, more homogeneous CD expression in tumor cells of early stages (Dukes' A and B) may be in keeping with previous investigations demonstrating higher CD activity in colorectal tumor tissues in the earliest clinical stages and in those from smaller tumors [39,43]. Thus it can be speculated that this lysosomal protease may facilitate the malignant progression of colorectal cancer by acting at an early stage of its histogenesis [39]. In this context, recent studies suggested that CD may promote tumor cell proliferation by activating growth factors or by interacting with growth factor receptors (i.e., epidermal growth factor, insulin-like growth factor 1), sharing a common IGF-II/mannose-6-phosphate transmembrane receptor [44–46]. Furthermore, CD is also considered to be a putative mitogenic factor and to be correlated with the amplification of the c-myc oncogene [47].

TABLE II. Cathepsin D Expression in Colorectal Adenocarcinoma Cells in Comparison With Patients' Survival

Patients' survival	Cathepsin D expression carcinoma cells		*P* value concerning patients' survival
	Positive expression	Negative expression	
>5 years	16	13	$0.05 < P < 0.10$
<5 years	9	22	
Total	25	35	

TABLE III. Cathepsin D Expression in Stromal Cells of the Colorectal Adenocarcinomas in Comparison With Patients' Survival

Patients' survival	Cathepsin D expression in stromal cells		*P* value concerning patients' survival
	Positive expression	Negative expression	
>5 years	16	13	$0.05 < P < 0.10$
<5 years	25	6	
Total	41	19	

The association of CD expression with the early stages of the disease and, subsequently, with better patients' survival has been reported in previous assays in gastric cancer [32,33]. Although a decreased frequency of CD expression at tumor cells was observed in the advanced stages of the colorectal carcinomas (Dukes' C and D), the intensely stained malignant cells were mainly located at the advancing margin of the invasion front in those specimens. The less aggressive, lower stage colorectal tumors, showing a better biological behavior and phenotype, may preserve the functional integrity of the biochemical pathways and synthesizing systems that are responsible for CD expression. These biological properties may be preserved only in the tumor clones comprising the invasion margin of the advanced stage tumors. Those phenotypically different cell populations may contain a mutant form of CD that may accentuate their aggressive biological behavior. Although neither amplification nor rearrangement of the CD gene (located on human chromosome 11p near the H-RAS proto-oncogene) has been found, future research may show the presence of a mutant form of this enzyme [48,49]. The different pattern of CD expression in early and late stages of the disease may also reflect tumor-associated impairment of the processing and secretion of the enzyme, or may be the result of an altered CD-mediated catabolic degradation of proteins or hormones [29,50].

The heterogeneity of tumor cell populations may explain to a certain extent the fact that lymph node metastatic foci from 10 cases were CD positive, whereas no

TABLE IV. Cathepsin D Expression in Colorectal Adenocarcinoma and Stromal Cells in Comparison With Patients' Survival

Cathepsin D expression	Patients' survival		*P* value concerning patients' survival
Carcinoma cells and stromal cells	>5 years	<5 years	
(+) and (+)	4	4	$P > 0.10$
(+) and (−)	12	5	$P > 0.10$
(−) and (+)	12	21	$P < 0.05$
(−) and (−)	1	1	$P > 0.10$
Total	29	31	

such expression was detected in the respective primary tumors. The tumor cell population that is responsible for the development of metastases may consist of monoclonal blast cells with specific biological properties that are reflected by biochemical markers (i.e., CD). Nevertheless, this expression may be enhanced by a specific local microenvironment different from that of the primary lesion [33].

A prominent finding in this study was the increased incidence of CD immunopositivity in the stromal cells of advanced Dukes' stage (C and D), as well as its correlation with a worse prognosis. The immunohistochemical method has the advantage of indicating exact tissue localization of the antigen. In such a study, Tetu et al. [27] provided evidence that expression in cancer cells may not be involved in the invasive phenotype of breast cancer, but the enzyme derived from the reactive stromal cells may have this important role. In vitro assays by Johnson et al. [50] and some other clinical studies [41,51] concluded that the increased CD activity in stromal components, such as infiltrative inflammatory cells and not the cancer-related synthesis of the enzyme, may have a more significant biological role. Although it is known that inflammatory cell infiltration at the border of the invasive front acts as a defense mechanism against tumor invasion, inflammatory cells of the stroma inside and adjacent to the tumor mass contain various enzymes including CD that could destroy the tissue architecture leading to the easier tumor spread. Graf et al. [25] reported that cathepsins discharged from inflammatory or carcinomas cells may activate other proteases. Subsequently, the degradation of the extracellular matrix by such proteases may provide an effective microenvironment for the proliferation and invasion of the carcinoma cells.

Furthermore, the distribution of the CD expression in these different cell populations, e.g., neoplastic cells and stromal inflammatory cells, raises the question of a possible influence between them via the presence or absence of one or more tumor-related diffusible factors [33,40]. It is also unclear whether the CD detected within stromal cells corresponds to CD synthesized within the recruited phagocytes of the tumor, or to CD engulfed by macrophages after its release by carcinoma cells, especially in the advanced stages of the disease where high intensity and concentration of the enzyme was observed in cancer and stromal cells of the invasive front of the tumor [33,52]. Recent research data support the positive correlation between CD and CD 31 expression, which is a marker of angiogenesis in breast cancer [53]. Increased CD secretion may be one of the events indirectly involved in angiogenesis, which constitutes a critical component of the metastatic process.

A principal finding of this assay was the identification of patients with colorectal cancer that expressed CD in stromal cells, but with no such expression in cancer cells. These patients had a worse overall prognosis and may constitute a subpopulation that warrants more aggressive postoperative follow-up and chemotherapy. Finally, the study of the significance of CD in cancer becomes more challenging since molecular biology studies show that new antiestrogen agents such as TCDD inhibit the CD gene expression [54].

Few studies have been conducted regarding the expression of this putative marker in colorectal cancer. Analysis using larger patient populations and different methodologies are worthy of future study, whereas the biological, functional, and prognostic role of CD remains to be further elucidated.

CONCLUSIONS

This study confirms the presence of CD in tumor and stromal cells in colorectal adenocarcinoma. The increased CD expression in tumor cells of early stages of the disease may facilitate the malignant progressing of colorectal cancer by acting at an early stage of histogenesis. Increased incidence of CD immunopositivity was observed in the stromal cells of advanced Dukes' stage carcinomas. Expression of CD immunoreactivity by the stromal cells of the tumor may be associated with a more invasive phenotype, and patients with CD overexpression in stromal cells of their carcinomas exhibit a worse 5-year overall survival. CD expression in tumor and stromal cells seems to have different functional roles and influences on colorectal adenocarcinoma progression, and its use as a marker may have prognostic and therapeutic implications.

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